

## **EXHIBIT 2**

**Potent induction of apoptosis by anthelmintics in human lung cancer cells:**

**involvement of wild-type p53 and p21 kinase inhibitor<sup>1</sup>**

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Running Title: Bendimidazole-induced apoptosis

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The abbreviations used are: MZ, (methyl 5-benzoylbenzimidazole-2-carbamate; FZ, methyl 5-(phenylthio)-2-benzimidazole carbamate (fenbendazole); TUNEL, terminal-deoxynucleotidyltransferase (TnT)- mediated dUTP-biotin nick end labeling; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate-buffered saline; Hoechst 33342, benzidine; NSCLC, non-small cell lung cancer; DMSO, dimethylsulfoxide; SSC, standard saline citrate; SDS, sodium dodecyl sulfate; MOPS, morpholinepropanesulfonic acid; PI, propidium iodide.

## Summary

We have studied the effect of the broad-spectrum anthelmintic benzimidazoles on the regulation of apoptosis in the human lung cancer cell lines. In this study, the in vitro effect of the benzimidazole compounds fenbendazole and mebendazole, on human lung cancer cell lines was determined. These drugs dramatically inhibited the growth of lung cancer cells in culture. Western blot analysis done using specific antibodies against Bcl-2, Bcl-xL, Bax, RB, cdc2, Cdk2, cyclin A, cyclin D, and p53 showed that treatment with fenbendazole and mebendazole did not alter the levels of any of these proteins except p53. The drug treatment also induced a dose- and time-dependent nuclear accumulation of wild-type p53 whose kinetics correlated well with the induction of apoptotic cell death. The effect of these benzimidazoles was further assessed in a number of human cell lines. Interestingly, only cell lines containing the wild-type p53 were highly sensitive to growth inhibition and apoptosis after benzimidazole treatment. The presence of wild-type p53 correlated well with enhanced growth arrest, micronucleation, and p53-dependent apoptosis in drug-treated cells. In addition, p53, MDM2 and p21<sup>Cip1/WAF1</sup> protein levels significantly increased by 24h after drug treatment. However, cell lines carrying mutated p53 were quite resistant to the cytotoxic effect of the drugs. Restoration of wild-type p53 function made tumor cells more sensitive to FZ and MZ induced apoptosis. The ability of benzimidazole to induce apoptosis in HeLa and SiHa cell lines, which express HPV-E6 protein as a dominant negative factor for p53-mediated cell death, was diminished. Thus, our collective findings strongly suggest that a p53-dependent mechanism contributes to the cytotoxicity induced by benzimidazoles in human cancer cells.

Benzimidazoles are broad-spectrum anthelmintics that display excellent activity against parasitic nematodes and, to a lesser extent, cestodes and trematodes (1). Bendimidazoles are effective antiprotozoal agents and have antifungal activity (2). It is currently believed that benzimidazoles exert their cytotoxic effects by binding to the microtubule system and disrupting its functions (3)(4). The suggestion that tubulin is a target for benzimidazoles has been supported by the results of drug-binding studies using enriched extracts of helminth and mammalian tubulin (3). Moreover, competitive drug-binding studies using mammalian tubulin have shown that benzimidazoles compete for colchicine binding and inhibit the growth of L1210 tumor cells in vitro (5)(3). However, benzimidazoles are selectively toxic to nematodes when administered as anthelmintics but are not toxic to the host (1). In contrast, benzimidazoles suppress the in vitro polymerization of mammalian tubulin (2). Differences in the affinity between host and parasite macromolecules for benzimidazole (6)(7) as well as the pharmacokinetics of benzimidazoles between the host and the parasite have been suggested as the factors responsible for the selective toxicity of benzimidazoles (8) but the actual molecular basis of this selective toxicity remains unclear.

Of all the proteins whose loss of function is associated with cancer development, p53 is the best known. In its wild-type form, it may function as a critical regulator of genotoxic stress and apoptosis (9)(10). Studies of the wild-type protein have shown that DNA damage or oxidative stress can increase the cellular accumulation of this protein by increasing its stability in stressed cells (10)(11)(12). Increases in the level of the p53 protein may in turn directly facilitate DNA repair, or they may indirectly inhibit cell-cycle progression or induce apoptosis (10)(12). Conversely, loss of p53 function may allow damaged cells to survive and permit DNA damage to accumulate, further promoting cellular transformation (11)(13)(14)(15). Therefore, genotoxic stress surveillance and concomitant p53 accumulation are important primary processes in damaged cells. In the study described here, we exposed a number of human lung cancer cell

lines of differing p53 status to benzimidazoles to evaluate the cytotoxicity of the drugs and found that the frequency of apoptosis was greater in the benzimidazoles-exposed cells. We also correlated the involvement of the p53 gene with the degree of drug sensitization and found that cell lines containing a functional p53 gene were more sensitive to benzimidazoles.

Benzimidazoles drugs are commercially available and show remarkable safety when used as anthelmintics in the treatment of many veterinary and human helminthiases. It is therefore possible that these drugs could be used clinically to inhibit the growth cancer cells. Our results demonstrate that BZs rapidly induces the production of the wild-type p53 protein in human lung cancer cell lines and concomitantly induces apoptosis.

## Experimental Procedures

**Materials**-Methyl-5-benzoylbenzimidazole-2-carbamate (mebendazole [MZ]) and methyl-5-(phenylthio)-2-benzimidazolecarbamate (fenbendazole [FZ]) were purchased from Sigma Chemical Company (St. Louis, MO). All other chemicals were purchased from Sigma unless otherwise indicated.

**Cell Culture**-Non-small cell lung cancer (NSCLC) cell lines were used in our studies. All except A549 were gifts from Drs. Adi Gazdar and John Minna (The University of Texas Southwestern Medical Center, Dallas, TX). All other cell lines were obtained from the American Type Culture Collection (Rockville, MD). In all cases, cell lines were grown according to the directions provided by suppliers. All culture media were supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Inc.) and antibiotics (100 mg/ml of streptomycin and 100 IU/ml of penicillin BRL). Mebendazole and fenbendazole were dissolved in dimethylsulfoxide (DMSO) and then diluted in phosphate-buffered saline (PBS) (1:1). When reagents containing DMSO were used, an equal volume of DMSO was added to the control cells.

**In vitro cell culture and proliferation assay**-All cell lines were grown in RPMI 1640 medium supplemented with 5% fetal bovine serum and 5% CO<sup>2</sup>. All experiments were done when the cells were 70% confluent. For cell growth measurements, 5x10<sup>4</sup> cells were plated in each well of six-well plates. Control cells and cells treated with benzimidazoles (0.05 µg/ml) were trypsinized and counted using a hemocytometer. Experiments were done in triplicate, and the mean and standard deviation were determined by standard methods. Using the Curve Fit 1.3 program, the 50% growth inhibitory concentrations (IC<sub>50</sub>) were extrapolated from a plot of the percent of control cell growth (triplicate determinations) versus drug concentration after 24 h of treatment.

**RNA Isolation and Northern Blot Analysis**-Total RNA was isolated from subconfluent cultures using the guanidinium thiocyanate method (3). After this, 20 µg of the RNA was electrophoresed in a denaturing 1.2% agarose/morpholinepropanesulfonic acid (MOPS)-

formaldehyde gel, transferred onto a nitrocellulose membrane, and hybridized to  $^{32}\text{P}$ -radiolabeled p53 cDNA probes, as described elsewhere (16). The  $^{32}\text{P}$ -labeled probes were generated using random primers ( $>8 \times 10^8 \text{ cpm}/\mu\text{g}$ ). Blots were washed at 65°C in 2 $\times$  standard saline citrate (SSC) for 30 min and then washed twice at 60°C in 0.1% sodium dodecyl sulfate (SDS) and 0.1X SSC. The cDNA probes used were 1.2-kb human p53 cDNA and an 800-bp fragment of human p21 cDNA.

**Antibodies**-The following antibodies were used: mouse anti-p21 monoclonal antibody WAF-1(Ab-1) Oncogene Sciences (Cambridge, MA); mouse monoclonal anti-Cyclin A (Sigma St Louis, MO), rabbit antihuman cyclin D (Upstate Biotech. Inc.) mouse anti-RB monoclonal antibody (Pharmingen , San Diego, CA), and mouse anti-c-myc monoclonal antibody (Invitrogen, Carlsbad, CA). Mouse anti-BCL-2 (100) monoclonal antibody, rabbit anti-Bcl-xL (S-19) polyclonal antibody, rabbit anti-Bax (N-20) polyclonal antibody, mouse anti-MDM2 (SM P14) monoclonal antibody, mouse anti-2 (100) monoclonal antibody, mouse anti-p53 (Bp53-12) monoclonal antibody, mouse anti-Cdk-2 (M2-G) goat polyclonal, antibody, and mouse anti-Cdc-2 p34 monoclonal antibody were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG were purchased from Amersham International (Arlington Heights, IL).

**Nuclear Staining Assay**- Cells were seeded onto chamber slides and treated with various reagents, after which cell monolayers were washed twice with ice-cold PBS (pH 7.4). Thereafter, cells were fixed for 5 min at 20°C in 10% formalin. The PBS washing step was then repeated once. To stain the nuclei, the cells were incubated for 10 min with 10  $\mu\text{g}/\text{ml}$  of Hoechst 33342 and then washed with PBS. Coverslips seeded with the stained cells were mounted in 80% glycerol in PBS containing 1 mg/ml P-phenylenediamine and examined with a Nikon epifluorescence microscope.

**Apoptotic assay and TdT FACS analysis**-Apoptotic assay was done using M30 CytoDEATH

apoptotic cell death assay kit ( Boehringer Mannheim ). Cells were grown on chamber slides, control and benzimidazole treated cells were stained with Mouse monoclonal antibody(clone M30) as per manufacturer's instructions. Apoptotic cells were examined with a Nikon microscope and photomicrographed. For TdT FACS analysis control and treated cells were collected by trypsinization, washed in PBS, and fixed overnight in 70% ethanol. The next day, cells were rehydrated in PBS for 30 min, centrifuged, and resuspended in PBS. For DNA analysis, propidium iodide (PI) was added at 50  $\mu$ g/ml, and the cells were incubated in the presence of RNase A (15 mg/ml for 30 min at 37°C). To detect DNA strand breaks associated with apoptosis, cells were fixed in 1% formaldehyde for 15 min at 4°C, rinsed in PBS, and stored at 4°C in ice-cold 70% ethanol. Before staining, the cells were washed in PBS, and  $10^6$  cells were resuspended in 50 ml of cacodylated buffer containing 100  $\mu$ ml TdT enzyme and 0.5 nM biotin-16 dUTP for 30 min at 37°C. Cells were washed in PBS and resuspended in 100 ml of 4X SSC containing 2.5 mg/ml fluoresceinated avidin, 0.1% Triton X-100, and 5% dry fat milk and then incubated at room temperature for 30 min in the dark. Finally, cells were washed in PBS and resuspended in PI buffer. Flow cytometry was carried out in a fluorescence-activated cell sorter (Epics Elite; Coulter, Inc., Hialeah, FL).

**Immunohistochemical Staining**-Cells were seeded onto glass coverslips and fixed as described above. The cells were blocked at 37°C for 30 min with 2% bovine serum albumin, 5% fetal bovine serum, and 5% normal goat serum in PBS. The cells were then incubated at room temperature for 45 min with anti-p53 (Ab-2) antibody (1:1000 dilution) in blocking buffer and washed with PBS. The cells were then incubated with HRP-conjugated goat anti-mouse IgG secondary antibody (Amersham). After washing, the immunocomplex was detected by an avidin-biotin complex kit, and slides were mounted as described above.

**Cell Lysates and Immunoblotting**-Cells were grown in 6-cm dishes, cultured, and treated as described above. To prepare the whole-cell lysates, the medium was removed. Then, cells were

washed twice with ice-cold Tris-buffered saline (TBS) (150 mM NaCl, 10 mM Tris; pH 7.6) and lysed with 0.5 ml of lysis buffer (10 mM Tris, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride [PMSF], 2 mM benzamidine, 10 mg/ml aprotinin, 10 mg/ml leupeptin, and 1 mM sodium orthovanadate) for 15 min. The lysed cells were then transferred to 1.5-ml microtubes and centrifuged at 15,000  $\times$  g for 10 min at 4°C. Then, the supernatants were collected, mixed with Laemmli's sample buffer, and subjected to western blot analysis as described elsewhere (5). Blots were probed with anti-p53 monoclonal antibody B p53-12 (Santa Cruz Biotechnology, Inc.), and immunocomplexes were detected using an Enhanced Chemiluminescence kit (Amersham) according to the manufacturer's directions. Blots were then reprobed, this time with anti-actin monoclonal antibody (Amersham), to show that protein loading was equal. After that, blots were again reprobed, this time with anti-WAF monoclonal antibody (Pharmingen, San Diego, CA).

**Pulse-Chase Experiments**-Cells were treated with 0.05  $\mu$ g/ml fenbendazole or mebendazole for 24 h. After this, control (mock-treated) and drug-treated H460 cells were incubated with 10  $\mu$ g/ml cycloheximide in drug-free medium for different times and then processed to obtain total cell extracts. Finally, samples were denatured by boiling them in SDS-loading buffer (100 mM Tris, pH 6.8; 2% SDS; 0.1% bromphenol blue; 10% glycerol; 25  $\mu$ M  $\beta$ -mercaptoethanol) and loaded onto a 10% SDS-polyacrylamide gel. Blots were probed with anti-p53 monoclonal antibody B p53-12 (Santa Cruz Biotechnology, Inc.) and anti-actin monoclonal antibody (Amersham). The immunocomplexes were detected using the Enhanced Chemiluminescence kit according to the manufacturer's directions as described above. The intensities of the bands were quantitated with a Phospholmager using ImageQuantTM Software (Molecular Dynamics, Sunnyvale, CA).

## Results

**BZ-induced Apoptosis in Human NSCLC Cell Line H460**-We tested the cytotoxic effect of fenbendazole and mebendazole on the human NSCLC cell line H460. After treatment with fenbendazole (0.05 µg/ml) or mebendazole (0.05 µg/ml) for 48 h, the cells became rounded and loosely attached to the plates, suggesting the cells were viability. Further analyses of the cell samples showed that the cells were undergoing apoptosis. Most of the morphological hallmarks associated with apoptosis were detectable, including cell shrinkage, DNA fragmentation, and chromatin condensation (Fig. 1A). The DNA strand breaks typical of the apoptosis, were also demonstrated by TUNEL and benzimidine staining (Fig. 1, A and B). The morphological changes produced by FZ and MZ treatments were indistinguishable. Gross morphological changes associated with a loss of viability were observed by in 24h after treatment, whereas signs of apoptosis, detected by DNA staining and TUNEL assays, became apparent by 24 h after drug treatment. Because of the close structural resemblance between FZ and MZ (i.e., both have the benzimidazole core structure), it is not surprising that they both induced apoptosis and the nuclear accumulation of p53 (Fig. 1C).

**Induction of Nuclear Accumulation of p53**-Both fenbendazole and mebendazole showed an apoptotic effect on the H460 cells. To rule out the possibility that fenbendazole and mebendazole were affecting other proteins known to play a role in various apoptotic pathways, we evaluated the effect of these drugs on a panel of such proteins. Many of these proteins are known activators or suppressors (e.g., Bcl-2, Bax, RB) of apoptosis and have already been shown to be expressed in these cells. Specifically, the levels of these proteins were evaluated by western blot analyses following fenbendazole and mebendazole treatments. Although the levels of nuclear p53, p21, and MDM2 were enhanced, there were, interestingly, no changes in the levels of Bcl-2, cyclin A, cyclin D, Cdc 2, Bcl-xl, Bax, RB, or Cdk-2 (Fig. 2). The protein level of the phosphorylated form of RB also remained unchanged after treatment for 24 h (Fig. 2).

Because fenbendazole and mebendazole specifically increased the amount of nuclear p53 detectable in the 460 cell line (Fig. 3), the apoptotic effect of these drugs on these NSCLC cells was presumably p53 dependent. There was also a positive correlation between the ability of fenbendazole and mebendazole to induce apoptosis and the ability of these agents to mediate the nuclear accumulation of p53. As a result of p53 protein accumulation, the p53-regulated genes were also expressed at much higher levels (Fig. 3). MDM2 and p21 levels analyzed by western blot analysis after fenbendazole treatment increased.

***Kinetics of Induction of p53 and Apoptosis***-The effect of fenbendazole and mebendazole on the nuclear accumulation of p53 appeared to be gradual and was not significant within the first hour of exposure. However, it became significantly detectable at about 16 h and peaked at 24 h (Fig. 4A). The appearance of p53 in response to drug treatment also coincided with the initiation of apoptosis, which was detectable by 24 h. Northern blot analysis, however, revealed no significant changes in the p53 mRNA levels (Fig. 4B), indicating that the nuclear accumulation of p53 was not due to an increase in p53 transcripts. As evidence that the p53 induced in these cells was functional, the level of the transcript for one of the p53 target genes, p21/WAF1, was significantly increased at the time the p53 level peaked (Fig. 4B).

The induction of p53 became detectable at a dose of 0.01  $\mu$ g/ml fenbendazole (Fig. 5A). However, the induction appeared to occur abruptly, which suggests the presence of a cooperative mechanism. Similar observations were made under serum-free conditions, suggesting that the source of the cooperative effect was not factors in the serum (data not shown). As predicted in light of the northern blot experiment, the steady-state p21 level (in parallel with the p53 protein level) did increase significantly over that in the control experiment in the H460 cells but not in the mutant p53-carrying H322 cells. The results of the DNA fragmentation assay, which assessed the effect of fenbendazole in inducing of apoptosis, and the assays of nuclear accumulation of p53 correlated well (Fig. 5B).

**Increased Half-life of p53 in Drug-treated Cells**-Because fenbendazole and mebendazole did not seem to affect the transcriptional rate of the p53 gene, we evaluated the effect of these agents on the stability of p53 in H460 cells and noted that both agents were able to prolong the half-life of the p53 protein in H460 cells. Because the results were similar for fenbendazole and mebendazole treatments, only data obtained from fenbendazole-treated cells are presented. Specifically, the half-life of the wild-type p53 in untreated H460 cells was about 6-8 h compared to 24 h in the drug-treated cells (Fig. 6). The former finding is consistent with the previously reported half-life of p53 in a number of cancer cell lines, which far outstripped the 20-to 30-min half life seen in normal fibroblasts (17).

***Selective Induction of Apoptosis by Fenbendazole and Mebendazole in Tumor Cells***

**Carrying Wild-type p53**-Studies to determine the IC<sub>50</sub> for fenbendazole and mebendazole were performed on a panel of six different human cancer cell lines: two carrying the wild-type p53 gene and actively expressing the p53 protein, (H460 and A549); one marked by homozygous deletion of the p53 gene and lacking p53 gene expression (H358); two expressing mutant p53 (H322 and H596); and one in which wild-type p53 is inactivated by human papilloma virus E6 protein. Comparison of the concentrations of bendimidazoles necessary to inhibit the growth of the different cell lines by 50% (IC<sub>50</sub>) indicated that wild-type p53 containing cell lines were 2-to 7-fold more sensitive than the p53-mutated or deleted cell lines (Table I). A dose of 166 nM (~ 0.05 mg/ml) was chosen for these studies because it had been shown previously that this concentration was sufficient to induce wild-type p53 after 24 h of treatment.

To further investigate the relationship between the induction of functional p53 and the subsequent apoptosis mediated by these drugs, we posited that if BZs require wild-type p53 in order to exert their effect, then fenbendazole and mebendazole would not be cytotoxic to the many tumor lines producing a mutated p53 protein. To investigate this possibility, human cell lines derived from NSCLC origins of differing p53 status were further analyzed by a 5-day cell

growth assay. Results for three cell lines (H460, H322, H1299) are shown in Fig. 7. H322 is a human lung adenocarcinoma line that produces a mutant p53 protein. The mutant p53 protein is generally more stable (18)(19) and this was reflected in the present case by the presence of higher amounts of the p53 protein in the nucleus of H322 cells prior to drug treatment (Fig. 7). H1299 is a p53 gene-deleted human NSCLC cell line and does not express any p53 protein. However, fenbendazole and mebendazole induced nuclear accumulation of p53 only in the H460 cell line, which carries wild-type p53 genes. Fenbendazole and mebendazole therefore appeared to be significantly effective in killing wild-type p53-containing cancer cells.

An analysis of 18 human tumor cell lines (Table II) was performed, which showed that these drugs had an effect on nuclear accumulation of p53 only in the cell lines carrying the wild-type p53 gene. Both drugs induced some degree of growth inhibition; rather than apoptosis, in cell lines that contained mutated or deleted p53; however, they induced greater growth inhibition in cell lines containing wild-type p53: As expected, HeLa and SiHa cervical cancer cell lines containing wild-type p53 along with HPV-E6 protein were less sensitive to the inductive effect of these drugs on apoptosis and p53 accumulation.

In order to further confirm that bendamidazoles works through p53 mediated pathway the synergistic effect of Ad5p53 and Fenbendazole on tumor cell growth was examined in four human lung cancer cell lines that differed in p53 status but were all transduced with Ad5p53: H1299 (*p53* deleted), H322 (*p53* mutated), H460 (wt *p53*) and A549 (wt *p53*). Because our initial dose-response studies indicated that 0.05 ug/ml FZ induced high levels of wt *p53* protein expression in H460 cells without toxicity, we used this concentration for all our proliferation assays. In those assays, growing, cultured cells were trypsinized and plated ( $10^4$  cells/well) and then infected the next day with Ad5p53 at 1 MOI. Viral supernatant was then added, after which cells were incubated for 24 h, washed with PBS, fed fresh medium or incubated with medium containing FZ for 24 h, washed again, and fed fresh medium. In contrast, uninfected cells

(controls) were treated with FZ for 24 h, washed with PBS, fed fresh medium, and then subjected to a 3-day growth assay (Fig.8A).

Our experimental results indicated that d1312 (empty vector) had no effect in combination with FZ (Fig. 8B). When the four lung cancer cell lines were transiently infected with 1 MOI Ad5p53 for 24 h, no growth suppression was observed, regardless of *p53* status. When all four lines were treated with FZ alone for 24 h, the *p53*-mutated and deleted cells were not growth inhibited, whereas the wt *p53* H460 cells was significantly so. However, a striking growth inhibition was observed in all four cell lines when the Ad5p53-transduced cells (1 MOI, 24 h) were treated with FZ for 24 h and were grown in normal medium for a 3-days growth assay. Our findings suggest that transducing of Adp53 will induce efficient *p53*-mediated killing of tumor cells in the presence of *p53* superinduction by FZ. The A549 cells (containing wt *p53*) showed 11% apoptotic cells following FZ treatment. Low dose wild-type Adp53 had no apoptotic effect, whereas the A549 cells showed 30% apoptotic cells death 48 h after combination treatment, as shown by terminal deoxynucleotidal transferase (TDT) staining analysis via fluorescence-activated cell sorting (FACS) as shown in Fig. 8C. These results suggest that FZ works though a *p53* dependent pathway.

***Inhibition of apoptotic effect by the dominant negative factor E6***-To provide more direct evidence that the cytotoxic effect of fenbendazole and mebendazole correlates with the availability of functional *p53* in the cell, we studied HeLa and SiHa ovarian cancer cells line that produce the E6 protein, which acts as a dominant negative factor for wild-type *p53*. This production of E6 is attributable, at least in part, to a dominant negative mechanism involving the degradation of the endogenous wild-type molecule. In this experiment, the HeLa (HPV-18) and SiHa (HPV-16) cells were treated with fenbendazole for 48 h, and the total cell proteins were subjected to immunoblot analysis for *p53* protein production. However, no induction of *p53* production was observed (Fig. 9), suggesting that the E6-mediated degradation of *p53* was

utilizing a path different from that used in normal cells. Because these results could also have been due to a decrease in the MDM2 protein level after fenbendazole treatment, we reprobed the membrane with MDM2 monoclonal antibody. However, MDM2 protein production remained low in both control and fenbendazole-treated HeLa and SiHa cells, whereas MDM2 production in the H460 cells increased several times with the increase in the p53 protein levels. Further, in these dominant negative lines, the level of the p21 protein did not increase above the base level after treatment with these drugs. This clearly showed that fenbendazole-mediated cell killing had become less effective in cells producing the wild-type p53-inactivating E6 protein, thereby limiting the ability of fenbendazole and mebendazole to mediate apoptosis. This finding may, in part, explain why killed those cells containing wild-type p53. However, one cannot rule out the possibility that a pathway other than the p53 pathway is also involved in the cytotoxic effect of these drugs.

## Discussion

We observed that the BZs fenbendazole and mebendazole selectively induced apoptosis in human NSCLC cells. Moreover, several lines of evidence in our study suggested that this apoptotic effect was mediated, at least in part, by the p53 protein. First, the kinetics of the induction of apoptosis and p53 accumulation were similar. Second, these compounds failed to induce apoptosis in mutant p53 cell lines and were also ineffective in inducing p53 production. Third, the production of an E6 protein acting as a dominant negative factor in the production of the wild-type p53 protein was sufficient to counteract the cytotoxic effect of these drugs. Finally restoration of wild-type p53 function by an adenoviral vector made tumor cells more sensitive to FZ and MZ induced apoptosis.

It is widely believed that restoring or enhancing wild-type p53 functions in tumor cells may one day be used to successfully treat many human cancers (20). Certainly, the *p53* gene is the most commonly mutated gene in human cancer (21)(22), and the resultant mutation of the p53 protein often inactivates tumor suppressor function, even though 40-50% of tumors may still retain copies of the wild-type *p53* gene. Recent evidence further suggests that, despite the production of functional wild-type p53 protein in human cancer cells, the amounts of wild-type p53 protein produced are so low that the protein, appears unable to execute its normal apoptotic function. In an effort to activate its apoptotic functions, it would therefore be of interest to determine the effect of fenbendazole and mebendazole on the trafficking of p53 protein between the cytoplasmic and nuclear compartment in these cells. Because phosphorylation of Ser 15 has been implicated as a mechanism underlying the increased stability of the p53 protein, the effect of these anthelmintics on the phosphorylation pattern of p53 is currently being investigated in our laboratory.

It is already known that DNA-damaging agents such as etoposide and Adriamycin induce production of the p53 protein in cell lines harboring the wild-type gene. These agents are

thought to enhance the level of p53 in cells by increasing its stability (23). This increase in stability has been borne out by immunofluorescence studies in fenbendazole-treated cells, which showed that there was a strong increase in p53 staining in the nucleus. Interestingly, our experiments also showed that the anthelmintic drugs we studied increased the half-life of p53 in H460 cells from 6 to 24 h.

The kinetics of p53 induction in H460 cells by these drugs and by MG132, a well-known proteosome inhibitor, are similar (data not shown). This strongly suggests that BZs inactivate a selective degradation pathway, thereby triggering the inductive effect on p53 in these cells. Therefore, it remains a possibility that the molecular target for BZs is not a kinase but a point in the p53-proteosome pathway of protein degradation where they can interfere with its progression.

This is the first report demonstrating that anthelmintics regulate the apoptotic function of wild-type p53 in human NSCLC cells. The fact that structurally related BZ analogues share this property of inducing apoptotic activities suggests that there is a unique and specific structural determinant of apoptosis embedded in their chemical structure and that this effect is mediated through wild-type p53. It has been suggested that the role of p53 in cell-cycle arrest may be distinct from its role in apoptosis and that each of these functions may be served by discrete domains in the molecule (24)(25). For example, a mutation in the p53 protein that inactivates its function in apoptosis may not necessarily affect its function in cell-cycle arrest. Interestingly, however, we observed in the present study that the induction of p53 protein in the drug-treated cells paralleled the induction of G1 cell-cycle arrest and apoptosis. It has been reported that Bax is a target gene for p53 (26). However, unlike p21, in the NSCLC wild-type p53 cell lines we studied here, the level of Bax protein, remained unchanged and was not upregulated by p53. Nevertheless, this result is consistent with the finding in some cell types that p53 does not appear to increase Bax levels (27).

The BZs are a group of structurally similar compounds that have been shown to possess antimitotic activity in vitro and in vivo (28) but also to induce a presumably p53-independent mechanism. In addressing this contradiction, we found no changes in the steady-state level of Bcl-2, RB, and Cdk-2 in H460 cells upon treatment with BZs for 24 h. However, it is possible that BZs regulate some of these proteins in a cell-type-dependent manner. In fact, the list of proteins that seem to play important roles in various apoptotic pathways is growing rapidly. However, whether BZs can regulate apoptotic genes other than those we have studied remains to be investigated.

In our study, fenbendazole and mebendazole induced p53 accumulation but failed to induce apoptosis in E6-expressing (HeLa and SiHa) cells. This inability may be attributed to the specific ubiquitination of E6-AP and the subsequent degradation of wild-type p53, actions that perhaps protect these cells from the effects of BZs. Alternatively, this inability may suggest the presence of an additional downstream step in the apoptotic pathway. To distinguish between these two possibilities, more human cancer cell lines carrying the wild-type p53 genes need to be evaluated.

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#### Figure Legends

**Fig. 1. Induction of apoptosis.** H460 cells were treated with 0.05 mg/ml mebendazole or fenbendazole and their morphological changes associated with apoptosis identified. A, widespread loss of viability was noted by gross examination of the cells at 24 h after treatment (upper panel). The cells were photographed using a phase-contrast light microscope. Chromatin condensation was noted under a fluorescence microscope at

24 h after treatment the condensation was shown by staining the nuclei with Hoechst 33342 fluorescent dye) (middle panel). DNA strand breaks were detected at 24 h after treatment using a direct immunoperoxidase method (lower panel). *B*, induction of apoptosis was assessed by TdT FACS analysis in the human lung cancer cell line H460. Cells were harvested after 24 h of exposure to fenbendazole and mebendazole (0.05 µg/ml) and the apoptotic cells quantitated after TdT staining as described in Experimental Procedures.

**Fig. 2. Effect of drug treatment on the level of various proteins.** Western blot analysis of various proteins in H460 cells before and after exposure to 0.05 µg/ml fenbendazole or mebendazole for 24 h. Whole-cell extracts were used for the analysis.

**Fig. 3. Ability of fenbendazole and mebendazole to induce the expression of p53 protein and its target genes.** *A*, H460 cells were treated with fenbendazole or mebendazole for 24 h (0.05 µg/ml), and the proteins from both control and treated cells were analyzed for p53 protein and p53 target gene expression. Increased p53 expression correlated with enhanced p21 and MDM2 protein levels. *B*, chemical structures of mebendazole and fenbendazole.

**Fig. 4. Fenbendazole-and mebendazole-induced expression of p53 protein and mRNA.** *A*, cell extracts were prepared from H460 cells exposed to fenbendazole (0.05 µg/ml) for various times (1 to 24 h). p53 protein was detected by immunoblot analysis. The position of the p53 protein is indicated by an arrow. *B*, northern blot analysis of p53 and p21 mRNA levels in H460 cells treated with 0.05 µg/ml fenbendazole or mebendazole after 24 h of treatment. Total RNA was extracted and used (20 µg per lane) for the

analysis.

**Fig. 5. The effect of fenbendazole in inducing apoptosis.** *A*, immunoblot analysis of p53 protein in the H460 human lung cancer cell line. The proteins were extracted from cells treated with different concentrations of fenbendazole and mebendazole for 24 h. ■ - represent FZ treated ; ▲- represent control untreated cells. *B*, NSCLC cell lines of differing p53 status were treated with 0.05 µg/ml fenbendazole for 24 h and then stained with p53 monoclonal antibody. The nuclear accumulation of wild-type p53 was associated with nuclear fragmentation that resulted in apoptosis.(A, C, and E) Untreated control cells and fenbendazole Z-treated cells (B, D, and F); H460 (wild-type p53)—upper panel; H322 (mutated p53)—middle panel; H1299 (deleted p53)—lower panel

**Fig. 6. Effect of fenbendazole and mebendazole on stability of the p53 protein.** *A*, stability of the p53 protein in H460 cells upon fenbendazole and mebendazole treatment. H460 cells were treated with 0.05 µg/ml fenbendazole for 24 h, and then both untreated control and treated cells were washed with PBS and treated with 25 µg/ml cycloheximide. After that, cells were harvested at different times. The total cell protein was extracted and analyzed on a 10% SDS-polyacrylamide gel, followed by western blot analysis using p53 and actin monoclonal antibodies (actin served as an internal control). The experiment was repeated twice, producing similar results both times. *B*, p53 protein stability in cells. Data from the pulse-chase experiments were quantitated with a Phospholmager using ImageQuantTM software. Circles and triangles represent data from fenbendazole-treated and control samples, respectively.

**Fig. 7. Effect of fenbendazole and mebendazole the induction of cell growth and nuclear accumulation of wild-type p53 protein.** A, The NSCLC cell lines H460 (wild-type p53), H322 (mutant p53), and H1299 (deleted p53) were exposed to fenbendazole (filled bar) or mebendazole (hatched bar) at concentrations of 0.05 µg/ml. The cells were plated onto six-well plates and their viability determined by trypan blue extrusion. Cells were then counted on a hemocytometer. The values shown are the means ± standard deviation of triplicate samples. Duplicate experiments gave similar results. Using equal amounts of protein extracts taken from each cell line before and after treatment, an immunoblot analysis of nuclear p53 was also performed to determine whether these drugs induced the production of p53. The upper band corresponds to p53, and the lower band corresponds to actin protein, which was used as an internal loading control.

**Fig. 8 Synergistic effect of Ad5p53 and Fenbendazole.** Approximately  $10^4$  cells were seeded on tissue culture plates 24 h before drug treatment or adenoviral infection. A) Cell growth was measured in untreated controls, cells treated with 0.05ng/ml FZ and cells treated with the combination of Ad5p53 (1 MOI) and FZ . B) H322 cells were also treated with an empty adenoviral vector, dl312, in addition to Adp53 to show dl312 had no effect alone or in combination with FZ. C) A549 lung cancer cells were examined for apoptotic cell death 48 h after FZ and Adp53 treatment alone or in combination. Percent apoptotic cells were measured by TdT-FACS analysis.

**Fig. 9 Effect of E6 protein on the cytotoxicity of fenbendazole.** Production of p53, MDM2, and p21 proteins after fenbendazole treatment was examined in HeLa and SiHa cells and compared with the production of these proteins in H460 cells. The positions of the p53, MDM2, and p21 protein bands are indicated.

**Table I. Sensitivity of cancer cell lines to bendimidazoles**

Phenotype and cell lines	$IC_{50}$	
	Fenbendazole (nM)	Mebendazole (nM)
<b>p53 positive</b>		
H460	152	106
A549	123	130
HeLa (p53 inactivated)	853	400
<b>p53 mutated</b>		
H322	816	871
H596	643	601
<b>p53 deleted</b>		
H358	654	893

<sup>a</sup>Concentrations of drugs (in nanomolars) required to inhibit growth by 50% after 1 day of exposure.

**Table II. Effect of fenbendazole and mebendazole on p53 induction and apoptosis in human tumor cell lines of differing p53 status<sup>a</sup>**

Cell Line	Tumor origin	p53 status	% Cell Viability		Nuclear p53	p53 Induction
			fenbendazole <sup>b</sup>	mebendazole <sup>b</sup>		
MCF-7	Breast	Wild-type (29)C	43.88 ± 7.3	24.26 ± 8.4	No	Yes
H460	Lung	Wild-type (30)C	39.16 ± 6.4	25.23 ± 7.6	Yes	Yes
H549	Lung	Wild-type (30)C	46.43 ± 13.3	39.19 ± 10.7	Yes	Yes
H322	Lung	R248H (30)	65.92 ± 4.9	68.50 ± 3.7	Yes	No
H596	Lung	R245C (30)	86.71 ± 11.5	86.19 ± 8.0	Yes	No
H226Br	Lung	R254 (31)	80.06 ± 11.1	64.24 ± 0.2	Yes	No
H1299	Lung	Deleted (30)	89.17 ± 1.1	89.18 ± 4.3	No	No
H358	Lung	Deleted (30)	88.35 ± 17.8	73.45 ± 1.8	No	No
Saos-2	Osteosarcoma	Deleted (32)	76.48 ± 5.9	65.36 ± 10.1	No	No
Hep 3B	Liver	Deleted (33)	79.49 ± 12.5	83.24 ± 1.5	No	No
SW480	Colon	R273H (21)	70.59 ± 7.6	63.63 ± 13.3	Yes	No
MDA 231	Breast	R280K (34)	65.26 ± 5.0	66.24 ± 15.8	Yes	No
SK-OV-433	Ovarian	Wild-type (35)C	52.52 ± 13.2	28.15 ± 6.1	Yes	Yes
HeLa	Cervical	Wild-type but inactivated by E6 (34)C	93.63 ± 7.4	92.42 ± 6.27	Yes	No

SiHa	Cervical	Wild-type but inactivated by E6 (36)C	94.52 ± 7.4	91.32 ± 8.6	Yes	No
RD a	Rhabdomyosarcom	R248W (37)	78.73 ± 12.0	72.24 ± 2.8	Yes	No
HT1080	Osteosarcoma	Wild-type (38)C	54.52 ± 12.0	44.49 ± 7.7	ND <sup>d</sup>	ND <sup>d</sup>

<sup>a</sup>The viability of the cells was measured by trypan blue extrusion cell count assay. The 100% value was derived from measurements obtained from untreated cells. Experiments were done in triplicate. p53 protein was examined by immunoblot analysis of the nuclear extracts isolated from each cell line before and after treatment with 0.05 mg/ml fenbendazole and mebendazole for 24 h.

<sup>b</sup>Concentration of fenbendazole and mebendazole = 0.05 mg/ml.

<sup>c</sup>This cell line carries a wild-type allele.

<sup>d</sup>ND, not done.